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Study of Acute and Subacute Action of Iron–Molybdenum Nanocluster Polyoxometalates

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Abstract—There were no significant deviations from the norm in the functional state of the liver, kidneys, and pancreas in the study of the acute toxicity of iron–molybdenum buckyballs intended for targeted drug delivery. No accumulation of nanoparticles or deviation from the norm in any investigated parameter was detected in the study of subacute toxicity.

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INTRODUCTION

Targeted drug delivery using nanocontainers is one of the actively developing areas of nanotechnology [1, 2]. Compounds with the structure of keplerates (buckyballs or fullerenes) and unified under the name polyoxometalates (POMs), which were first synthesized by Professor A. Müller (University of Bielefeld, Germany) [3, 4], are promising for use as nanocontainers. POMs possess the following advantageous characteristics: solubility in water and the ability to absorb reversibly various organic compounds [5], form complexes [6], and move under the influence of weak electric fields [5].

An iron–molybdenum buckyball $Mo_{72}Fe_{30}$ is one of the most typical representatives of the class of buckyballs [4].

$$[Mo_{72}Fe_{30}O_{252}(CH_3COO)_{12}\{Mo_2O_7(H_2O)\}2\{H_2Mo_2O_8(H_2O)\}(H_2O)_{91}] \approx 150H_2O.$$

The presence of an internal cavity and "windows" through which the exchange of water and different substances can occur in the structure of this keplerate makes its application as a nanocontainer possible. Unlike other prospective nanocontainers (carbon fullerenes) [7], POMs are able to decompose, forming simpler compounds of molybdenum and iron [5, 6], which further participate in the metabolic processes natural for the organism.

In particular, molybdenum takes part in the oxidation–reduction reactions as a cofactor of oxidases. Despite the absence of cumulation of soluble molybdenum compounds, its chronic excessive intake can be accompanied by the deposition of uric acid and urates [8, 9], which cause the symptoms of arthragra [8, 9]. The accelerated formation of uric acid is due to the activation of hypoxanthine oxidase and xanthine oxidase molybdenum-dependant enzymes, which catalyze the oxidation of purine bases [8]. The excessive intake of iron, in contrast to that of molybdenum, damages various organs owing to the deposition of hemosiderin. Because of the participation of iron in the Fenton reaction, free radical oxidation is triggered.

Because the effects of nanosized materials can differ from the effects of the element constituting these particles, a thorough investigation into the toxicity of buckyballs is required.

Thus, the aim of this work is to investigate the acute and subacute toxicity of iron–molybdenum buckyballs intended for use as containers for targeted drug delivery.

MATERIALS AND METHODS

The synthesis of $Mo_{72}Fe_{30}$ was carried out according to the literature procedure [4]. The preparation of $Mo_{72}Fe_{30}$ nanocluster was carried out in two steps, and nanocluster $(NH_4)_{42}[Mo^{VI}_{72}Mo^{V}_{60}O_{372}(CH_3COO)_{30}(H_2O)_{72}] \cdot 300H_2O \cdot 10CH_3COONH_4(Mo_{132})$ was an intermediate product [3]. Starting reagents in the synthesis of Mo_{132} were chemically pure grade ammonium heptamolybdate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pure-for-analysis-

grade hydrazine sulfate $N_2H_4 \cdot H_2SO_4$, chemically pure grade ammonium acetate CH_3COONH_4, chemically pure grade glacial acetic acid CH_3COOH, and medical-grade ethanol 95%. For the synthesis of $Mo_{72}Fe_{30}$ from Mo_{132}, iron chloride (III) hexahydrate $FeCl_3 \cdot 6H_2O$ Panreac (assay 97–102%), pure-for-analysis-grade sodium acetate $Na(CH_3COO) \cdot 3H_2O$, high purity grade hydrochloric acid HCl, and pure-for-analysis-grade sodium chloride NaCl were used. The synthetic methodologies involve the washing of the intermediate compound and final product, upon which the admixtures of starting materials, which are more soluble in the washing conditions compared to the nanocluster polyoxometalates themselves, were removed. In order to control the quality of washing, particularly from the residual iron chloride, the washing liquid was analyzed for the absence of the chloride ions. The synthesized compounds Mo_{132} and $Mo_{72}Fe_{30}$ were characterized, and their characteristics were compared with the literature data by an elemental analysis method using an autoanalyzer Carlo Erba CHNS-OEA1108 for a C, N, H and atomic emission spectrometer with inductively coupled plasma iCAP-6500 Duo (Thermo Scientific) for molybdenum and iron; by a dynamic laser light scattering method using a universal suspension analyzer Brookhaven Zeta-Plus/B1 90 with a wavelength of 659 nm in relation to the size of the nanoclusters; by structural characteristics using a Spectrum-BX II Fourier IR spectrometer (PerkinElmer) in KBr pellets, a spectrophotometer Helios-α, and a Raman spectrometer Triplimate 21. The intermediate compound Mo_{132} was analyzed for the absence of toxic admixtures like hydrazine by NMR spectroscopy using a Bruker DRX-400 NMR spectrometer; 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard and the samples were dissolved in D_2O. The intermediate and final compound $Mo_{72}Fe_{30}$ was also analyzed in the powdered condition for the absence of admixtures, including residual salts of molybdenum and iron, by X-ray analysis using a diffractometer D8 ADVANCE, Bruker (CuK_α emission).

The experiment for the investigation into acute and subacute toxicity was carried out on 95 outbred rats of both sexes and with body weights of 200–230 g, which were on a standard feed of the vivary. Housing conditions and treatment of animals used in the experiment corresponded the EU Council Directive of November 24, 1986, "On the Approximation of Laws, Regulations and Administrative Statutes of the EU on the Protection of Animals Used for Experimental and Other Scientific Purposes" (86/609EEC). The animals were divided into 12 groups. The first group consisted of intact rats (ten animals). In investigational groups 2–7 (ten rats each), the introduction of various dosages of nanoparticles was performed in three different ways. Groups 2c–7c (five animals each) were the control groups in relation to the investigational ones; the animals in these groups were administered distilled water in the same volumes and in the same ways as the rats in the investigational groups were administered buckyballs. The buckyballs were injected into the animals in groups 2 and 3 intramuscularly as a single dose of 0.15 mg/100 g. The animals were withdrawn from the experiment 1 and 24 h after injection, correspondingly. In group 4, the test material was introduced intraperitoneally, the dose was 0.15 mg/100 g, and the withdrawal from the experiment was performed after 24 h. In groups 5 and 6, the buckyballs were introduced intragastrically and the doses were 0.50 mg/100 g and 30 mg/100 g, correspondingly; the action of the nanoparticles continued for 24 h. In group 7, the animals were injected iron–molybdenum buckyballs intramuscularly for 30 days on a daily basis in the dosage of 0.15 mg/100 g.

It was established earlier that the area of acceptable over time stability for buckyballs is a weak acidic or neutral medium, and the best method for buckyball introduction, namely intramuscular injections, was chosen [10, 11]. The amount of molybdenum contained in one injection of buckyballs corresponded to the upper limit of normal for the daily intake of molybdenum, and the amount of iron was 21.5-fold less than its daily intake. The animals were withdrawn from the experiment by ether anesthesia. The content of molybdenum in the organs was determined using an atomic emission spectrometer with inductively coupled plasma iCAP-6500 Duo (Thermo Scientific) after the mineralization of samples.

Biochemical studies characterizing the cytolytic activity of the injected substances and their influence on the functional state of various organs were performed according to the "Guideline for the Experimental (Preclinical) Investigation of New Pharmaceutical Substances of the Ministry of Public Health of the Russian Federation" [12]. In the blood plasma, the activity of the following enzymes was determined: aspartate aminotransferase or AST (EC 2.6.1.1), alanine aminotransferase or ALT (EC 2.6.1.2), alkaline phosphatase (EC 3.1.3.1.), and α-amylase (EC 3.2.1.1.), as well as the content of glucose, creatinine and total protein. Vital diagnostics (St. Petersburg) ready-to-use reagents kits were used for the investigation. The optical density was measured using an SF-56 spectrophotometer.

The statistical analysis of the material was carried out using Statistica 6.0 (StatSoft, Inc.) and Microsoft Excel 2003 programs. A significance level of 5% ($P < 0.05$) was used upon the examination of statistical hypotheses.

RESULTS AND DISCUSSION

The qualification of the synthesized nanocluster $Mo_{72}Fe_{30}$ by a set of physicochemical methods revealed that its chemical composition and structure were in a good agreement with the literature data [4] and that it was of high purity.

Table 1. Biochemical parameters of the animal blood plasma on several days after the intramuscular injection of iron–molybdenum buckyballs (dosage 0.15 mg/100 g)

Parameter	2 I.M. single dose 1 h	2c Control for group 2	3 I.M. single dose 24 h	3c Control for group 3	7 I.M. multiple doses 30 days	7c Control for group 7
Glucose, mmol/L	5.9 ± 0.8	7.3 ± 0.3	8.0 ± 0.8	6.5 ± 0.4	7.4 ± 0.6	7.1 ± 0.2
AST, U/l	15.3 ± 0.6	13.6 ± 0.5	26.3 ± 1.5*	12.9 ± 0.5	15.4 ± 1.6	13.7 ± 0.6
ALT, U/l	10.4 ± 0.4	10.9 ± 0.8	9.6 ± 1.1	8.9 ± 0.4	10.0 ± 0.8	9.6 ± 0.5
AST/ALT	1.47 ± 0.06	1.28 ± 0.11	2.93 ± 0.47*	1.47 ± 0.10	1.55 ± 0.06	1.44 ± 0.10
Alkaline phosphatase, U/l	53.6 ± 4.2	48.9 ± 2.8	36.8 ± 4.7*	51.0 ± 2.5	44.3 ± 6.2	48.2 ± 3.5
α-Amylase, mg/(s L)	31.5 ± 2.2	33.1 ± 1.8	29.7 ± 1.7	33.9 ± 1.4	33.6 ± 4.6	29.7 ± 1.3
Total protein, g/L	65.7 ± 1.9	73.9 ± 2.0	68.2 ± 1.7	72.2 ± 2.7	68.6 ± 2.7	74.3 ± 2.4
Creatinine μmol/L	57.4 ± 1.2	66.4 ± 5.3	59.0 ± 2.9	62.3 ± 4.9	83.7 ± 5.6	69.4 ± 3.4

* Differences with the corresponding control group are statistically significant at $P < 0.05$.

Table 2. Biochemical parameters of the animal blood plasma 24 h after a single dosage of iron–molybdenum buckyballs in a number of ways

Parameter	4 I.P. 0.15 mg/100 g	4c Control for group 4	5 I.G. 0.50 mg/100 g	6 I.G. 30 mg/100 g	5c Control for groups 5 and 6
Glucose, mmol/L	9.1 ± 0.5	8.7 ± 0.6	7.8 ± 1.4	10.1 ± 1.0*	7.0 ± 0.3
AST, U/l	19.6 ± 1.1*	14.2 ± 0.5	21.6 ± 2.4*	24.1 ± 2.2*	14.0 ± 0.4
ALT, U/l	13.1 ± 1.6	9.9 ± 0.5	16.7 ± 3.1*	18.0 ± 2.6*	9.2 ± 0.6
AST/ALT	1.54 ± 0.10	1.44 ± 0.04	1.35 ± 0.21	1.36 ± 0.09	1.55 ± 0.09
Alkaline phosphatase, U/l	39.1 ± 2.8*	49.8 ± 3.0	48.5 ± 6.1	38.5 ± 2.6*	51.1 ± 4.0
α-Amylase, mg/(s L)	32.0 ± 2.0	32.9 ± 0.7	28.4 ± 2.8	31.3 ± 4.0	30.3 ± 3.7
Total protein, g/L	65.0 ± 3.8	71.2 ± 2.2	69.4 ± 10.3	65.9 ± 3.0	72.8 ± 2.2
Creatinine, μmol/L	63.6 ± 2.3	68.8 ± 5.0	58.8 ± 2.7	66.9 ± 3.5	68.6 ± 5.4

* Differences with the corresponding control group are statistically significant at $P < 0.05$.

In order to determine acute toxicity, a solution and a suspension of the iron–molybdenum buckyballs with the highest possible concentration were prepared and three ways for the injection of the buckyballs were chosen: intramuscular (I.M.), intraperitoneal (I.P.), and intragastric (I.G.). The concentration of the solution of the iron–molybdenum buckyballs for intramuscular and intraperitoneal injection was 1 g/L and corresponded to the maximum solubility of these nanoparticles. Taking into account the fact that the maximum liquid volume for parenteral infusion is 0.3 mL, the maximum dosage for groups of animals 1 and 2 was 0.15 mg/100 g. For rats, the admissible liquid volume for intragastric infusion is 1 mL; in this case, the buckyball load was 0.50 mg/100 g. In contrast to the parenteral administration, peroral administration allows introducing the suspension of buckyballs with the concentration of 60 mg/mL, which corresponds to a maximum dosage of 30 mg/100 g.

The administration of the maximum possible dose of buckyballs was not accompanied by any lethality; therefore, one can conclude that the investigational material is not highly toxic, because the amount of the nanoparticles (30 mg/100 g) was 200-fold higher than a proposed therapeutic dosage (0.15 mg/100 g).

The biochemical characteristics of the functional state of various organs were determined in the blood plasma of animals from all groups according to the guideline for the experimental (preclinical) investigation of new pharmaceutical substances [12].

The data on the biochemical measurements in rats from experimental groups after the administration of various doses of iron–molybdenum buckyballs in comparison with the same data for the control groups, in which animals were administered an equivalent amount of distilled water in three different ways, are presented in Tables 1 and 2.

As is seen from Table 1, there are no deviations in the investigated parameters from the control (group 2c)

Table 3. Biochemical parameters of the animal blood plasma after the injection of water in a number of ways

Parameter	1 Intact	2c 0.3 mL H_2O I.M. after 1 h	3c 0.3 mL H_2O I.M. after 24 h	4c 0.3 mL H_2O I.P. after 24 h	5c 0.3 mL H_2O I.G. after 24 h	7c 0.3 mL H_2O I.M. 30 days
Glucose, mmol/L	6.9 ± 0.1	7.3 ± 0.3	6.5 ± 0.4	8.7 ± 0.6*	7.0 ± 0.3	7.1 ± 0.2
AST, U/l	13.2 ± 0.7	13.6 ± 0.5	12.9 ± 0.5	14.2 ± 0.5	14.0 ± 0.4	13.7 ± 0.6
ALT, U/l	9.1 ± 0.5	10.9 ± 0.8	8.9 ± 0.4	9.9 ± 0.5	9.2 ± 0.6	9.6 ± 0.5
AST/ALT	1.46 ± 0.10	1.28 ± 0.11	1.47 ± 0.10	1.44 ± 0.04	1.55 ± 0.09	1.44 ± 0.10
Alkaline phosphatase, U/l	50.4 ± 3.0	48.9 ± 2.8	51.0 ± 2.5	49.8 ± 3.0	51.1 ± 4.0	48.2 ± 3.5
α-Amylase, mg/(s L)	29.7 ± 2.9	33.1 ± 1.8	33.9 ± 1.4	32.9 ± 0.7	30.3 ± 3.7	29.7 ± 1.3
Total protein, g/L	72.0 ± 2.7	73.9 ± 2.0	72.2 ± 2.7	71.2 ± 2.2	72.8 ± 2.2	74.3 ± 2.4
Creatinine, μmol/L	65.8 ± 6.2	66.4 ± 5.3	62.3 ± 4.9	68.8 ± 5.0	68.6 ± 5.4	69.4 ± 3.4

* Differences with the group of intact animals are statistically significant at $P < 0.05$.

1 h after the injection of nanoparticles (group 2). At the same time, 24 h after the intragastric infusion of buckyballs (group 6) there is a statistically reliable increase in the glucose content compared to the corresponding control group (Table 2). The increase in the glucose level, which is a nonspecific symptom of dyscrasia, can be due to both the very procedure for the introduction of intragastric probe and the toxic action of the increased amount of buckyballs (30 mg/100 g).

It was also observed that the activity of AST increased 24 h after the infusion of buckyballs by each of the three methods used (groups 3–6) and the activity of ALT increased after the intragastric infusion of nanoparticles (groups 5–6) relative to these same parameters in the control animals which were administered distilled water (Tables 1, 2).

In group 3, the activity ratio for the enzymes AST/ALT was also above the norm. The reproportion of the activities of aminotransferases in favor of AST points to the predominant increase in the permeability of the membranes of cardiomyocytes. The increase in the AST activity and de Ritis coefficient under the short-term action of the nanomaterial did not depend on the amount of the specimen introduced and was more pronounced in the case of intramuscular injection.

In ordered to elucidate the influence of the procedure for the injection of the buckyballs, the biochemical parameters of intact animals and rats from the control groups were compared (Table 3). A small statistically significant ($P < 0.05$) increase of the glucose content was only observed after the intraperitoneal injection of water compared to the same data for the intact animals (group 4c). In the rest of the control groups to the investigational groups 2, 3, 5, 6, and 7, the examined parameters did not differ statistically significantly from the level of the parameters for intact animals. Hence, dyscrasia, whose symptoms are abnormal glucose content and enzyme strength, was due to the action of the nanoparticles.

In groups 5 and 6, ALT activity increased 24 h after the intragastric injection of buckyballs (Table 2). It is likely that the activity of ALT, which is an indicator of liver damage, was influenced by the injection method (intragastric) under which the buckyballs can get to the liver faster compared to other injection methods. At the same time, the AST : ALT ratio in groups 5 and 6 did not statistically significantly differ from this coefficient for intact animals, which does not allow establishing the accurate localization of cytolysis.

The histological study of liver conducted earlier did not indicate any structural changes in hepatocytes after the 30-day action of buckyballs [11]; at the same time, the hyperemia of central veins and the veins of portal tracts provided evidence for the onset of the symptoms of an inflammatory process. In part of the liver blood vessels of rats from group 7, "sludge complexes" were detected, the formation of which is apparently due to the increase in the amount of thrombocytes, erythrocytes, and hematocrit values because of the repetitive intake of iron [12]. The total amount of leucocytes and nuclear Arneth count of the rats injected with buckyballs for 30 days did not differ statistically significantly from the parameters of the peripheral blood of intact rats, which gives evidence of the absence of an inflammatory process of the whole organism.

The absence of an increase in the activity of alkaline phosphatase, which is one of the indicators of cholestasis, in the blood plasma of all experimental rats points to the integrity of the cholepoietic function of hepatocytes after the short-term and long-term injection of buckyballs (Tables 1, 2).

Alkaline phosphatase is also a specific enzyme of osteoblasts and participates in mineralization, and the activity of this enzyme is an indicator of the speed change in the remodeling of mineral components in the bone tissue. It is a known fact that molybdates can reduce the activity of phosphatases, apparently, due to the interaction of molybdates with the thiol groups of

Table 4. The content of molybdenum in the whole blood of rats after the injection of buckyballs

Group	1 Intact	2 Intramuscularly 0.15 mg/100 g after 1 h	3 Intramuscularly 0.15 mg/100 g after 24 h
Molybdenum, μg/L	73.2 ± 0.1	477.5 ± 4.5*	86.6 ± 1.9*
Group	4 Intraperitoneally 0.15 mg/100 g after 24 h	5 Intragastrically 0.50 mg/100 g after 24 h	7 Intramuscularly 0.15 mg/100 g daily for 30 days
Molybdenum, μg/L	135.5 ± 0.5*	923.5 ± 3.5*	69.0 ± 4.7

* Differences with the group of intact animals are statistically significant at $P < 0.05$.

cysteine and imidazole groups of histidine located in the active sites of these enzymes [9].

In groups 3, 4, and 6, a negligible but statistically significant decrease in the activity of alkaline phosphatase was detected, which is probably due to the inhibiting action of molybdenum. A short-term—over 24 h—decrease in the activity of alkaline phosphatase eliminates the risk of the development of osteoporosis upon a single injection of buckyballs.

In a comparison of the mean values for other parameters in groups 2–6 (the activity of α-amylase and content of total protein and creatinine in the blood plasma), no statistically significant differences with the parameters of the intact and control rats were detected, which gives evidence of the absence of the pronounced changes in kidneys, liver, and pancreas of the animals from these groups. In group 7 (with the subacute injection of buckyballs), no statistically significant deviations from the norm for all tested biochemical parameters were detected either, which makes it possible to suppose the absence of the processes of cytolysis and the damage of kidneys, liver, myocardium, pancreas, and bone tissue after a daily injection of buckyballs.

An investigation into the molybdenum content in the blood of the animals after the injection of nanoparticles by different ways is presented in Table 4.

A statistically significant increase in the molybdenum content in blood after a one-time load with buckyballs, regardless of the method used for their injection, was noted. The amount of molybdenum detected in blood 24 h after the injection of buckyballs increased proportionately with the amount of the nanomaterial injected (groups 3, 4, and 5). A significant decrease in the molybdenum level in blood after 24 h (group 3) compared to the content of this microelement after 1 h (group 2) following the intramuscular injection and the normalization of this parameter after daily injections of nanoparticles over a period of 30 days (group 7) are in accordance with the literature data on the absence of accumulation of molybdenum [8, 9]. Earlier works also mark the absence of accumulation of molybdenum in liver, kidneys, skin, and bone tissue [10, 11]. Presumably, the decomposition of the nanoparticles and the accelerated excretion of the elements constituting the buckyballs promote the normalization of the parameters under examination (glucose, aminotransferase, and alkaline phosphatase), whose change was detected upon a single dosing of buckyballs.

CONCLUSIONS

1. The injection of the maximum possible dose of iron–molybdenum buckyballs (300 mg/kg) was not accompanied by the lethality of animals.

2. During the investigation into the acute toxicity of iron–molybdenum buckyballs, no pronounced changes in the examined parameters of the functional state of liver, kidneys, and pancreas was detected.

3. During the investigation into the subacute toxicity of iron–molybdenum buckyballs, the absence of accumulation of nanoparticles was established and no deviations from the norm for the examined parameters of the functional state of liver, kidneys, pancreas, myocardium, or bone tissue were found.

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